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ORIGINAL ARTICLE

Genotypic and phenotypic characterization of *Streptococcus mutans* strains isolated from patients with dental caries

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ABSTRACT

Background and Objectives: The oral cavity harbors numerous *Streptococcus mutans* strains which display remarkable genotypic and phenotypic diversity. This study evaluated the genotypic and phenotypic diversity of 209 *S. mutans* strains isolated from 336 patients with dental caries and compared with the universal reference strain, UA159.

Materials and Methods: Selective cultivation on mitis-salivaries-bacitracin agar and species-specific polymerase chain reaction (PCR) was carried out to isolate and identify the 209 *S. mutans* isolates from 336 patients with dental caries. Arbitrarily primed polymerase chain reaction (AP-PCR), PCR amplification of specific gene, acid production and biofilm formation capacity were performed to evaluate the genotypic and phenotypic variation. Student's t-test and Chi-square test were used for analysis of variables and a probability *(P)* of <0.05 was considered as significant.

Results: Our study revealed a high degree of genotypic and phenotypic variability among the clinical strains. We observed significant differences in colony morphology, generation time, biofilm formation, and acid production while growing in culture medium. All the clinical isolates were able to lower pH while growing in Todd-Hewitt broth. Consistent with phenotypic variations, we also observed genotypic variation by AP-PCR and gene specific PCR. AP-PCR analysis suggested that most of the patients with dental caries have distinct type of *S. mutans* strains. Genes related to various two component systems were highly conserved among the isolated strains, however, bacteriocin encoding genes such as *nlmAB, nlmC* were absent in nearly half of the clinical isolates.

Conclusion: Our results support that *S. mutans* clinical isolates have wide genotypic diversity and show variation in growth kinetics, acid production, acid tolerance and biofilm formation capacity and indicates the presence of diverse mechanism to initiate and establish the biofilm lifestyle which leads to tooth decay.

Keywords: Dental caries; *Streptococcus mutans;* Genotype; Phenotype; Virulence

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INTRODUCTION

The initiation and successful development of dental caries is caused by multiple bacterial and host factors, such as the composition and biochemical activity of the biofilm organisms, dietary habit, genetic constitution and tooth architecture (1-3). The mutans

streptococci, specifically *Streptococcus mutans*, are considered to be the primary causative agents of dental caries (4, 5). The ability to form biofilm on tooth surface, production of organic acid from various carbohydrates (acidogenicity), ability to survive at low pH (acidurance), outstanding ability to outcompete other bacteria by the production of bacteriocin and the adaptation to rapidly changing environment can be attributed as the major virulence factors of *S. mutans* (2, 6-8). Development of natural competence, which is coordinately regulated with the bacteriocin production, is another vital attribute that provides genetic diversity to *S. mutans* for niche adaptation and colonization (9).

Previously, several attempts have been made to correlate the caries incidence with certain genotypes of *S. mutans*, however, no correlation was observed among multiple studies from various geographic locations (10-12). Furthermore, no relationship was found between the caries status of an individual and the distribution of 41 putative virulence genes or genetic elements in 33 *S. mutans* isolates (13). Whole genome sequence analysis and comparison of the presence or absence of individual genes have demonstrated wide genetic variability among *S. mutans* strains. *S. mutans* strains also display phenotypic variability in accordance with the variation in their genetic repertoire (8, 12-15). This is especially important for *S. mutans*, which is naturally competent bacterium and therefore has the potential for rapid genome diversification through horizontal gene transfer (9). In a previous study, Palmer et al. observed a high degree of phenotypic variability among 15 of the completed draft genomes of 57 geographically and genetically diverse isolates of *S. mutans* (1). Nevertheless, current knowledges are not conclusive regarding diversity of *S. mutans* and caries status. Moreover, no studies are available in genotypic diversity and phenotypic characteristics of *S. mutans* strains with caries status from Bangladeshi population. The aim of the present study was to investigate the genotypic and phenotypic heterogeneity of 209 *S. mutans* strains isolated from 336 patients with dental caries from Bangladesh.

MATERIALS AND METHODS

Study population. Samples were randomly collected from the plaque surface of 336 unrelated patients of different age (from 15 to 67 years) and sex groups with dental caries from Dhaka, Bangladesh in this study. An informed written consent was taken from each participant. Patients with chronic diseases and who had taken antibiotic therapy for the last two weeks were excluded from the study.

Ethical approval. The study protocol for human subjects was approved by the Institutional Review Board of the Faculty of Biological Sciences, University of Dhaka (Ref. No. 82/Biol.Scs).

Bacterial culture and growth. Samples were collected from plaque surface of patients with dental caries using sterile toothpick and suspended in 1 ml phosphate buffer saline (PBS) buffer. 100 µl of the sample was spread on the mitis salivarius agar supplemented with 0.5 IU/mL bacitracin (MSB agar) (Sigma, USA) and incubated at microaerophilic condition at 37°C for 48 hours. *Streptococcus* strains were routinely grown in Todd-Hewitt medium (Hi-Media, India) supplemented with 0.2% yeast extract (THY) at 37°C. For the monitoring of growth, overnight cultures were diluted into fresh medium (1:100), grown to late exponential phase and absorbance was taken at 630 nm at various time intervals. Doubling time was calculated based on two OD values taken from the logarithmic phase of the growth by using the formula, $r = \ln [OD2/OD1]/(T2-T1)$ and represents the average value of at least two measurements. We have included first 40 strains (SN1 to SN40) for simplicity.

Identification of *S. mutans* strains. Colony morphology on the mitis salivarius-bacitracin agar medium (MSB) was primarily used for the selection of *S. mutans.* Presumptive *S. mutans* colonies were further verified by catalase test and hemolysis on blood agar plate and confirmed by PCR with previously reported species-specific primers targeting Smu.479, which encode DNA-directed RNA polymerase subunit omega, *rpoZ* (16). Species-specific PCR was performed by colony PCR. Briefly, a single colony was picked from the THY agar plate and the cells were suspended directly into PCR mixture in a microcentrifuge tube. The PCR assay included 30 cycles of denaturing at 95°C for 30 seconds, annealing at 50°C for 45 seconds and extension at 72°C for 1 min.

Typing of clinical isolates by AP-PCR. The ge-

netic diversity of *S. mutans* isolates was analyzed by arbitrarily primed PCR (AP-PCR) by using the primers set OPA 02 (5'-TGCCGAGCTG-3') and OPA 13 (5'-CAGCACCCAC-3') as described previously (17). The colony PCR was performed using 2× PCR master mix under the conditions of 35 cycles of denaturation at 94°C for 30 seconds, annealing at 32°C for 1.5 minutes, extension at 72 °C for 2 minutes, with initial denaturation at 95°C for 5 minutes, and a final extension at 72°C for 10 minutes. The amplicons generated by AP-PCR were then separated by 1.5% agarose gel electrophoresis. The molecular size of each band was calculated and a dendrogram was generated using the UPGMA cluster analysis and analyzed by using the Dice coefficient (>95%) in accordance with Mitchell et al. (18).

PCR amplification of virulence genes. The detection of *nlmA, nlmB, nlmC, comC, comD comE, gtfB, gbpA, vicK, ciaH, atp,* and Smu. 1906 was performed by colony PCR using primers specific to gene based on the UA159 genome sequence (13). In addition to the strains being tested, purified genomic DNA from *S. mutans* UA159 was used as a positive control and distilled water was used as a negative control in each PCR. *gyrA* gene was used as an internal control.

Investigation of the acidurity of *S. mutans*. The ability to withstand the acidic pH was investigated according to previously described methods with modification (12, 19). In brief, overnight grown bacterial cultures were diluted to 1:50 into THY broth as control or THY broth acidified with HCl (pH 5.0) in 96 well microtiter plate and incubated at 37°C and the growth was monitored for 24-hour at 630 nm using a microplate reader (Micro Read 1000, ELISA plate analyzer, Global Diagnostics, Belgium). Each experiment was performed at least three times.

Acidogenesis of *S. mutans* clinical isolates. Production of acid by metabolism of carbohydrate was evaluated as previously described methods with minor modification (12, 19). In order to investigate the acid production capacity of the clinical isolates, overnight grown *S. mutans* culture was inoculated in 10 ml of THY broth (pH 8.32) and the pH was determined at different time intervals (0 hr, 24 hr, 48 hr and 72 hr) using a pH meter. Each experiment was performed in triplicates and mean value is represented with standard deviation.

Biofilm assay. Biofilm assay was performed according to a previously described methods with minor modification (20, 21). In brief, overnight grown bacteria in THY broth were diluted 1:20 and inoculated into fresh THY medium supplemented with 1% sucrose into wells of polystyrene flat-bottom 24-well microtiter plate and incubated for 48-hr at 37°C in microaerophilic condition. After incubation, the culture medium was decanted, and the wells were washed three times with distilled water and stained with 1% crystal violet for ten minutes. The plates were further washed with distilled water twice to remove the unabsorbed dye. The cells were then resuspended into 1-ml 95% ethyl alcohol and absorbance was taken at 550 nm. Each experiment was performed in triplicates and mean value is represented here.

Statistical analysis. To examine whether mean of the quantitative variables of the strains is different from the reference value, one sample student's t-test was performed. Chi-square test was employed to assess the association between qualitative variables and the study groups (disease outcome). Correlation coefficient between two quantitative variables was also computed to know the strength of the relationship between them. For all tests, a probability *(P)* of <0.05 has been considered as significant. All statistical analysis was performed using the SPSS software version 20 (SPSS, Chicago, IL, USA).

RESULTS

Dental health analysis of study subjects. The DMFT (decay-missing-filled-Teeth) index is widely used to assess the epidemiology of dental caries status. The mean DMFT values of this study was 4.82 with standard deviation of 2.19 (n=336). The mean age of the study populations was 42.4 and 38.6 for male and female, respectively. Among the 336 patients, 61.6% was male and 38.4% female.

Isolation and identification of *S. mutans* clinical strains. Morphological and cultural characteristics of the isolates ranged from round size blue colonies with granular frosted glass appearance to round, blue, rough and shiny colonies (data not shown). There was also spherical form, raised or convex elevation ranging from a pinpoint to pinhead size with a rough surface, flat, light blue or dark colonies on MSB agar

plate. Pinpoint colony with granular, frosted glass appearance was primarily selected as *S. mutans.* All the strains displayed positive Gram staining reaction and catalase negative (data not shown). Colony PCR with species-specific primers further confirmed the isolates as *S. mutans* since the expected product size of SMU.479 was found in agarose gel after electrophoresis. The prevalence of *S. mutans* was 81.84% (275 of 336) in patients suffering from dental caries based on colony morphology and biochemical analysis. 209 of 275 colonies (76%) of the preliminary identified colonies were finally confirmed as *S. mutans* by specific-species PCR.

Genotypic diversity of *S. mutans* isolates. To get an idea about genotypic diversity of the isolated strains, we performed AP-PCR for all 209 *S. mutans* strains. Fig. 1a demonstrates the AP-PCR patterns carried out with OPA-02 and OPA-13 primers using some representative isolates, where we observed different spectrum of amplicons for each isolate, which indicates the high level of genetic polymorphism among the isolated strains. The results of AP-PCR analysis of the first 40 isolates revealed 10 different clusters and 26 different genotypes among the strains (Fig. 1b).

Growth kinetics of *S. mutans* clinical isolates. To investigate the growth kinetics of various clinical isolates, we performed the growth curve analysis for 12 hours. Compare to the reference strain UA159, we observed noticeable variation in growth rate in some strains, however, most of the strains grew at similar rate to UA159 (Fig. 2a and 2b). Some of the isolates demonstrated very slow growth rate with long dividing time (>150 minutes) and took three to four days to have distinct colony on the agar plate at microaerophilic condition. Growth pattern of the UA159 was in the middle among the isolated strains (dividing time 57 minutes). The mean doubling time of the isolated strains were 75.12 min (standard deviation of 24.92) with 95% confidence interval [14.81, 21.63].

Distribution of *S. mutans* putative virulence genes. Eight of the putative virulence genes (*gtfB, gbpA,atp, htrA, vicK, ciaH, comD* and *comE*) were present in all clinical isolates and five genes were differentially present among the isolates. The presence of bacteriocin encoding genes (*nlmAB* and *nlmC*) was observed in 63% and 48% of the isolates respectively (data not shown).

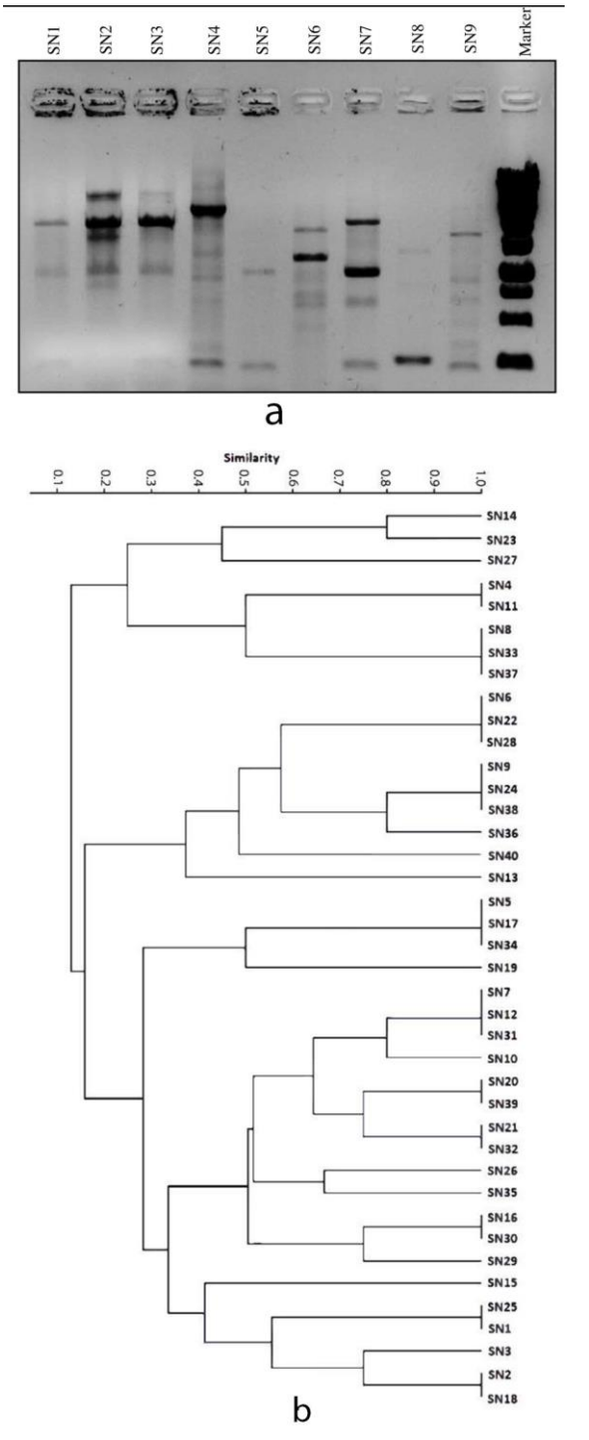

Fig. 1. (a) AP-PCR patterns of selected *S. mutans* isolates. Colony PCR was performed with the primer set OPA-02 and OPA-13 primers (lanes 1-9). Lane 10 contains the DNA ladder (1 kbp plus). (b) Dendrogram delineating the genetic diversity of the 40 isolated *S. mutans* strains. The Dice coefficient was computed based on UPGMA clustering algorithm.

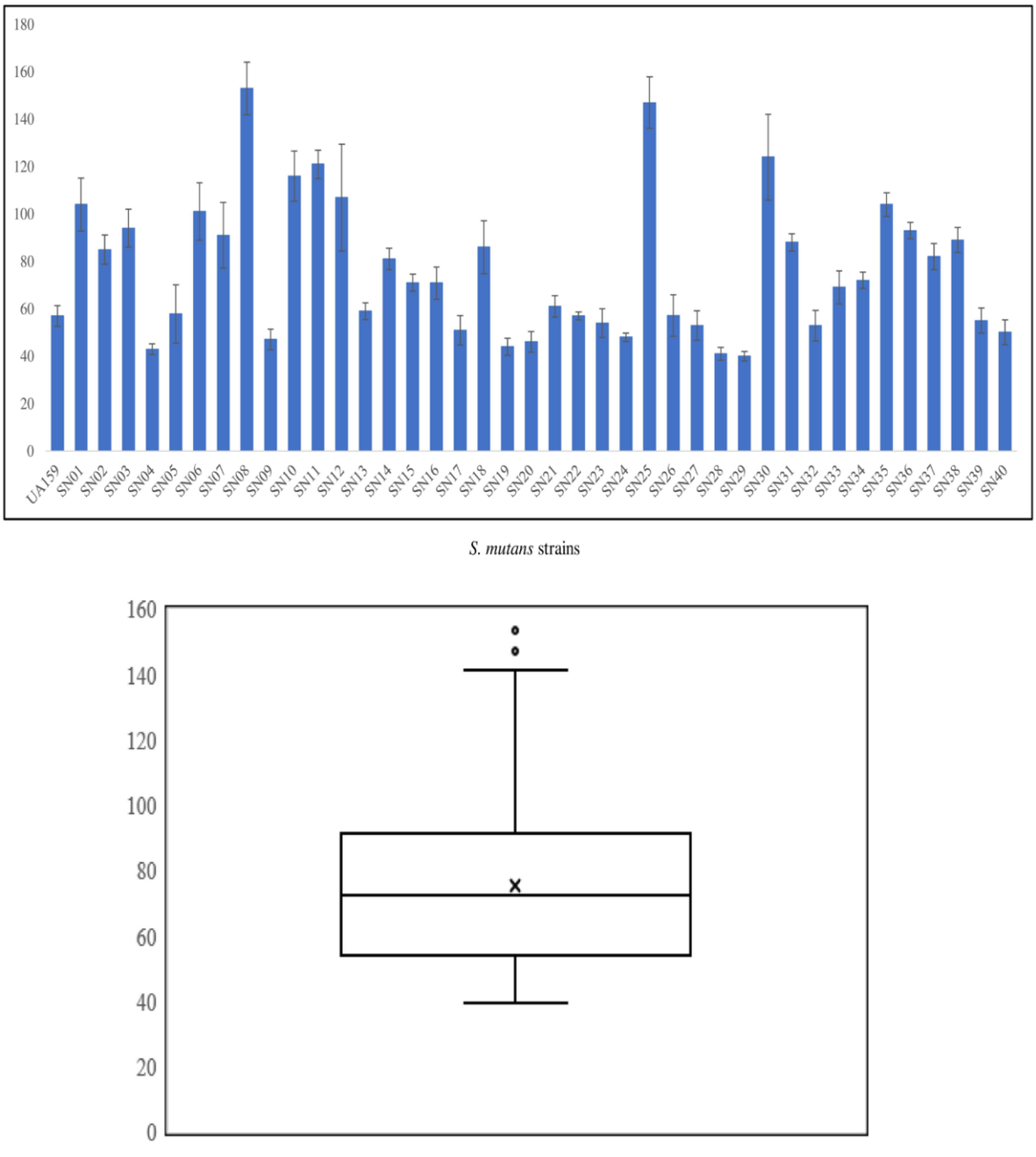

Fig. 2. (a) Mean doubling time of the isolated strains (SN01 to SN40). Doubling time was calculated based on two OD values taken from the logarithmic phase of the growth and represents the average value of at least two measurements. (b) Box plot showing the summary of the mean doubling times of the isolated strains (n=209).

Acid tolerance by *S. mutans* clinical isolates. We observed noticeable reduction in growth compared to growth at initial media pH of 8.3 for every strain (Fig. 3). While some strains faced difficulty to grow at pH 5.0, some strains grew faster than UA159. Mean growth of the clinical isolates at pH 5.0 for 20-hour at absorbance 630 nm was 0.66 with standard deviation of 0.22. The variation of the strains was significant (p<0.001; 95% CI= [-0.171, -0.112]).

Acidogenesis of *S. mutans* clinical isolates. We noticed that all of the clinical isolates have remarkable ability to produce acid while growing in THY broth (Fig. 4) and turned the initial pH of the media

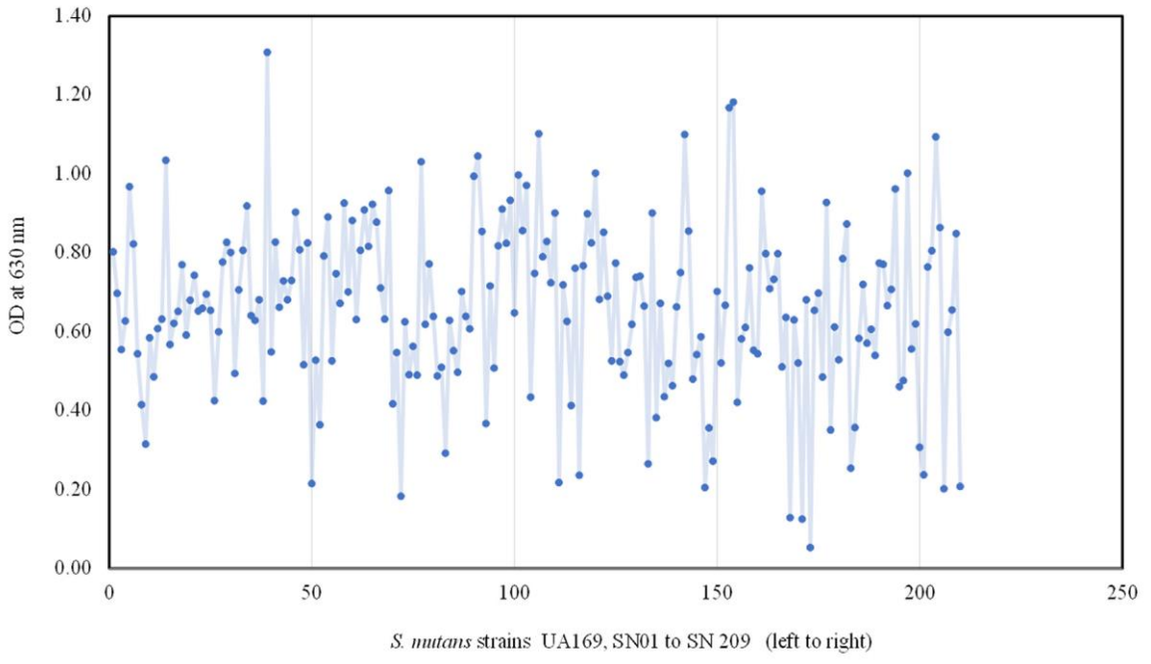

Fig. 3. Acid tolerance of the isolated strains. Isolated strains were incubated in THY broth either at pH 8.3 or pH 5.5 and growth was monitored for 24-hour period. Error bar represents the standard deviation.

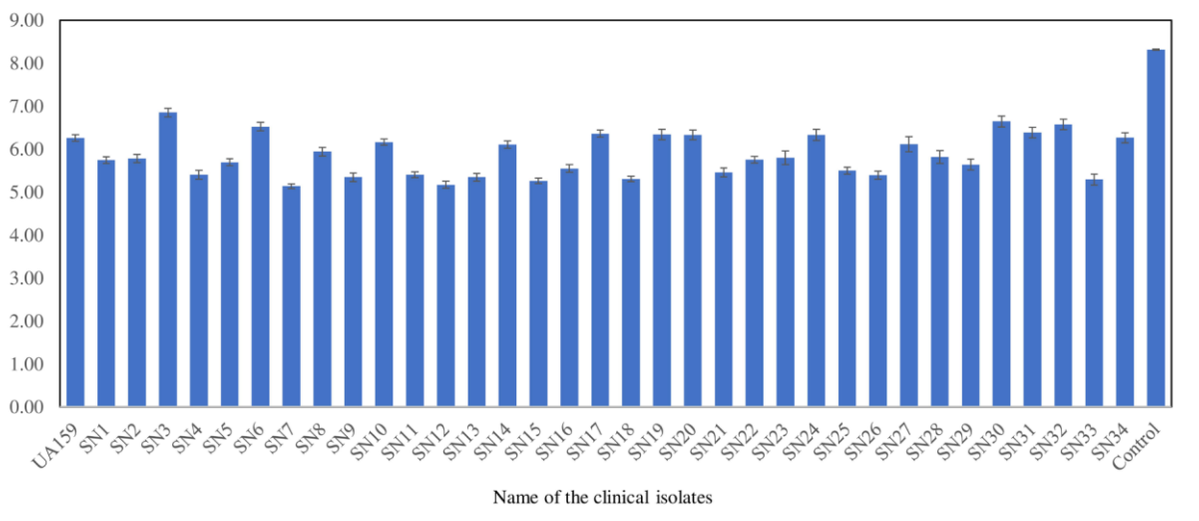

Fig. 4. Acid production by the isolated strains. Isolated strains were incubated in THY broth and the pH was measured every 24-hour intervals with a pH meter. Error bar represents the standard deviation.

from 8.34 to more acidic pH (up to pH 5.0). Most of the clinical isolates displayed better acid production ability than the reference strain, UA159 which has turned the THY broth from pH 8.34 to pH 6.25. Mean pH value after 72-hour growth was 5.7 with standard deviation of 0.355. Two-tailed analysis suggested significant variation among the clinical isolates (p<0.001, 95% CI= [0.637, 0.539]).

Biofilm forming capacity of *S. mutans* clinical isolates. Biofilm formation assay suggested that all the strains retained significant level of biofilm formation capacity as like UA159. However, variation in biofilm formation capacity was also present among the clinical strains and some of the isolates displayed superior biofilm forming capacity than UA159 (Fig. 5). The mean biofilm forming capacity was 0.521

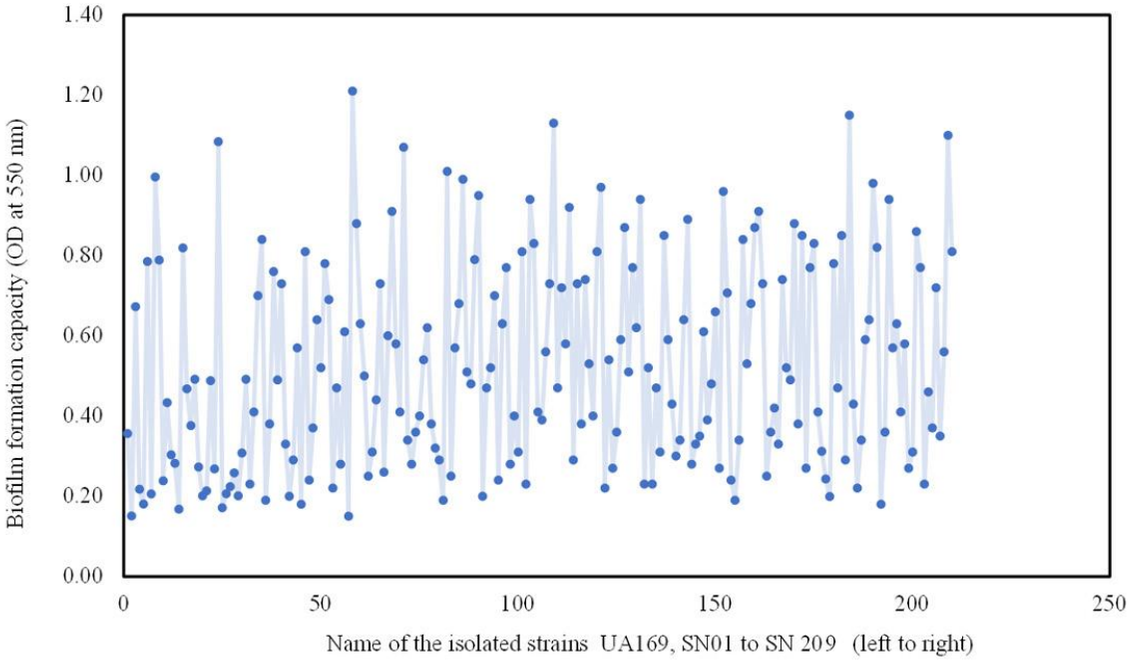

Fig. 5. Biofilm formation by the isolated strains. Bacteria were grown in THY medium supplemented with 1% sucrose for 48 hours in 24 well plates. After the growth, supernatants were discarded and stained with crystal violet and washed with distilled water. Cells were resuspended into ethanol and absorbance values were taken at 550 nm. Error bar represents the standard deviation. Each experiment was performed in triplicates.

with standard deviation of 0.256. Further statistical analysis revealed significant variations among mean of the strains with reference UA159 (P<0.001; 95% CI= 0.126, 0.196).

DISCUSSION

In this study, we showed that the phenotypic and genotypic properties that are associated with the virulence of *S. mutans* are diverse and vary significantly among 209 newly isolated clinical strains. We found that types and colony morphology of the isolated strains on MSB agar vary considerably from patient to patient. We observed multiple strains in the same sample with various colony morphology, which further confirmed that dental plaques indeed contain multispecies biofilm structures. We also performed AP-PCR of first 40 strains to investigate the genotypic diversity of the isolated strains and found that 26 different genotypes are present among the strains. High levels of genotypic variations were also found previously by several groups (1, 8, 15, 22). Zhou et al. classified 730 *S. mutans* isolates into 337 distinct genotypes by AP-PCR fingerprint analysis (23). The high prevalence of genotypic variations can be attributed to age of the study population, diversified horizontal gene transfer, various nutritional behavior, and chemical environment in the oral cavity. The presence of several genotypes of *S. mutans* strains in the dental plaque of an individual patient with distinct virulence potential implies the synchronized action of multiple virulence traits which augments the severity of caries development.

In our study we found that most of the strains are genetically homogenous for genes associated with two-component systems which is in accordance with a previous report which reported a significant level of genetic homogeneity among *S. mutans* strains (13). However, our results are in contrast with Palmer et al. who reported that wide variations exist among strains of *S. mutans* in the pathways involved in quorum sensing, genetic competence and non-lantibiotic bacteriocins (1). Variation in the same species is prevalent in several bacterial species and it was revealed that *S. mutans* strains differ significantly in individual gene level and distribution of virulence gene is not related with the caries status (24).

As like the genotypic diversity, our phenotypic studies revealed that isolated *S. mutans* strains have wide variation in phenotypic diversity. Some strains displayed better sucrose-dependent biofilm forming capacity than the universal reference strain, UA159 (Fig. 5) and some were crippled in biofilm forming capacity. Our results further support several previous reports that *S. mutans* clinical isolates vary widely in biofilm formation capacity (1, 12, 20). Biofilm formation capacity of *S. mutans* is aided by various genes, which encode several surface antigens to attach the teeth surface (25, 26). The variation in biofilm forming capacity can be due to the presence or absence of various biofilm associated genes, prevalence of polymorphism among these genes and differential epigenetic regulation. We also investigated the presence of biofilm associated gene, *gbpA* in the clinical isolates, however, this gene was present in all strains. This indicates that expression of pathogenic phenotype in the multispecies biofilm structure in the dental plaque and differential expression of virulence genes due to environmental pressure are pivotal in dental biofilm to cause dental caries.

Acidurity and acidogenesis are two most important phenotypic traits of *S. mutans* (8). Our results suggest that isolated strains have differential response to acid stress. Most of the clinical strains suffered from growth constraints and individual strain exhibited distinct growth kinetics at pH 5.0 (Fig. 3). Our results are in agreement with a previous study where significant variation in growth characteristics were observed during acid stress (1). In addition to acidurity, we also investigated the acidogenesis character of the isolated strains and found noticeable variation among acid production while growing on THY media and all the strains could turn the initial medium pH of 8.32 to more acidic pH from 5.02 to 6.54. Our results showed little variation from a previous study where equal acidogenicity was observed among the *S. mutans* isolates (20). Ability to produce acids from carbohydrate metabolism and to withstand low pH in the rapidly changing environment in the oral cavity confers *S. mutans* strains selective advantage to antagonize commensal organisms and associate with other organisms to form multi-species biofilm which helps in initiation and progression of dental caries.

The phenotypic and genotypic properties of *S. mutans* clinical isolates presented here imply that clinical strains have undergone intense evolutionary changes to cope up with the rapidly changing environment in the oral cavity. This study has helped us to better understand the cariogenic variations of *S. mutans* clinical strains which can be used to devise new approaches to control *S. mutans* mediated dental diseases thereby. Since biofilm formation and acid production are the key virulence factors of *S. mutans*, inhibition of biofilm formation by antagonistic beneficial bacteria (27) or antimicrobial peptide (28) and intake of low carbohydrate food to reduce the acid production can be efficient approaches to control dental caries problem worldwide.

Although the present study provides substantial evidence that *S. mutans* clinical isolates exhibit wide variation in genotypic and phenotypic diversity, it has several limitations. First, genotypic variation was based on AP-PCR and PCR amplification of individual gene in this study instead of whole genome sequence analysis which could provide more information on genotypic variations of the isolated strains. Second, only three colonies were primarily selected from the MSB plate for each sample. Therefore, we might have missed other important strains in this study. Finally, we have only focused on *S. mutans* strains, however, dental caries is caused by concerted activities of multiple bacterial species.

CONCLUSION

Taken together, our results indicated that *S. mutans* clinical isolates exhibit profound variations in both genotypic and phenotypic characteristics and further supported the role of biofilm formation, acid production and acidurity as the key virulence factors to cause dental caries. Further genomics and transcriptomics studies are warranted to get insight into the tremendous variation exists among the *S. mutans* clinical isolates and their possible interactions with symbiotic and antagonistic neighbors prevalent in the oral cavity.

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